

ISOLATION AND STUDY OF A RECOMBINANT CARBOHYDRASE  
XYLANASE FROM *BACILLUS LICHENIFORMIS*

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## ABSTRACT

Xylanases are hydrolytic enzymes involved in the degradation of xylan, the main component of plant biomass. In the present study xylanase, XynA, from Kazakh strain *Bacillus licheniformis* was obtained in recombinant form in *Escherichia coli* cells. The *xynA* gene was amplified from the genomic DNA of the *B. licheniformis* and cloned in pET-28c (+) vector under the control of the promoter of bacteriophage T7. Recombinant XynA was produced in ArcticExpressRP(DE3) cells by plasmid gene expression, protein purification was carried out by metal affinity chromatography. During the study, the dependence of the enzymatic activity of recombinant xylanase on temperature and pH was observed, it was established that xylanase has the highest activity at +50°C and pH of 6.9. The activity units at these values of temperature and pH were 1859 per ml of purified protein. The working temperature and pH ranges in which xylanase conserves more than 70% activity from maximum is 40-60°C and 5-8, respectively.

The data obtained are essential for the use of carbohydrase xylanase from *B.licheniformis* in biotechnological processes in the processing of vegetable raw materials.

**Keywords:** xylanase, *Bacillus*, recombinant enzyme, xylanase activity.

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## INTRODUCTION

Xylanases (endo-1,4-p-xylanase, EC 3.2.1.8) are enzymes with a molecular weight of 6-80 kDa, which carry out the hydrolysis of  $\beta$ -1,4-xylozide bonds in xylans (hemicelluloses), which is one of the main components plant biomass. The most studied microbial xylanases (from bacteria and fungi), which are involved in the biological degradation of plant biomass and thus play an important role in the cycle of organic carbon in nature [1,2]. Most of the known microbial xylanases belong to the 10th and 11th families of glycoside hydrolases [1].

In industry, xylanases are used for bleaching paper pulp in paper production, to improve the baking properties of flour, the technology of converting vegetable residues, obtaining biologics with prebiotic properties and to increase the nutritional value of feed for farm animals – the processing of cereals, straw, bran.

As it is known, xylan, like cellulose, is not digested by the digestive system of monogastric animals (pigs, poultry, rabbits) and the processing of feed with xylanase increases the content of oligo- and monosaccharides and increases the digestibility of the feed [2]. The literature presents

data on the use of xylanase for the treatment of grains and cereal crops as part of a complex of enzymes such as cellulose, cellobiohydrolase, catalase, phytase, peroxidase, oxidoreductase, laccase, esterase [3].

## Materials and methods

### Strains and plasmids

In this study *Escherichia coli*: DH5 $\alpha$ , ArcticExpressRP(DE3) strains were used. The bacterial strain *Bacillus licheniformis* T6 was taken from a laboratory collection (isolated from soil near the city of Taraz).

Plasmid pET-28c(+) (Novagen, UK) was used for cloning and expression.

### Oligonucleotides

In the present work, oligonucleotides synthesized in National Center for Biotechnology were used (table 1).

Table 1. Oligonucleotides

XynA-NcoI	5'-CATGCCATGGCTAGCCCAGACTACTGGCAA-3'
XynA-NotI	5'-TTTTCCTTTTGC GGCCGCCACACTGTTACGTTAGAACTTC-3'
T7 fw	5'-TAATACGACTCACTATAGGG-3'
T7 rv	5'-TAATACGACTCACTATAGGG-3'

### Media

Luria-Bertani low salt broth was used (1% tryptone, 0.5% yeast extract, 0.5% NaCl) for cultivation of *E.coli* cells. For incubation of the transformed cells, was used SOC medium (2% tryptone, 0.5% yeast extract, 0.05% NaCl, 2.5 mM KCl, 20 mM MgSO<sub>4</sub>, 20 mM glucose, pH7.5). Preparation of media was carried out according to the protocol of Maniatis [4].

### Cloning of a xynA gene into the expression vector

*XynA* gene was amplified from the DNA of *Bacillus licheniformis* using primers XynA-NcoI/XynA-NotI. The amplified gene was inserted in the expression vector pET-28c(+), both digested by NcoI and NotI restriction enzymes. For amplification and cloning, the previously described protocols were used [5]. The presence of the xylanase gene in the transformant strains was confirmed by PCR screening using primers T7 fw/rv. Plasmid DNA hydrolysis, dephosphorylation and ligation were carried out using the enzymes NcoI, NotI, FastAP, T4 DNA ligase and the corresponding buffers for them: Buffer Orange and T4 DNA ligase Buffer manufactured by Thermo Scientific. The nucleotide sequence of the *xynA* gene was determined by sequencing using primers T7 fw/rv.

### Expression of xynA gene in E.coli cells

Competent cells of the *E.coli* ArcticExpressRP(DE3) strain were transformed by the obtained plasmid vector pXynA by electroporation. The selection of transformants was performed on agar LB medium with kanamycin at a concentration of 50  $\mu$ g/ml. The selected colony-transformant was inoculated to 600 ml of Luria-Bertani broth in the presence of kanamycin and cultured at 37°C with shaking 150 rpm. Upon reaching an optical density of OD<sub>600</sub> = 0.6, an isopropyl- $\beta$ -D-thiogalactopyranoside was added at a final concentration of 0.5 mM. XynA protein induction was performed at + 37°C and shaking at 150 rpm for 16 hours.

### Purification of recombinant XynA xylanase

Bacterial cells were centrifugated (6000g, + 4°C, 7 min) and lysed with lysozyme (2 mg/ml), followed by ultrasonic sonication [5]. The XynA protein was isolated from the cell lysate by metal affinity chromatography using 1 mL HiTrap Chelating (General Electric) column activated with Ni<sup>2+</sup> ions. For the creation a linear gradient of the eluate, an AKTA Purifier 10

FPLC chromatograph (General Electric) was used. Imidazole was used as the eluate at a concentration from 20 to 500 mM with the addition of 500 mM NaCl in 20 mM Tris-HCl (pH 8.0).

### Obtaining of xylan

Crushed beech sawdust was poured with distilled water at the rate of 1 g of sawdust 40 ml of water. The mixture was autoclaved at +121°C and a pressure of 1 atm, 30 minutes. The liquid was removed and the alcohol-water extraction was performed. Sawdust was poured with a 1:1 alcohol/water mixture and heated with reflux refrigerator at +90° C for 1 h. The liquid was removed and the sawdust was washed with water and the alcohol-water extraction was repeated for 2 hours. After removing the liquid, the sawdust was washed with water and dried on the filter paper at +105°C for 1 hour. The precipitate was rinsed in a solution of 4% NaOH (+18°C), the resulting suspension was placed on a shaker at +18°C for 6 hours and filtered. The filtrate was neutralized with acetic acid to pH 5.5-6.5, alcohol was added in a ratio of 1:1 and incubated at +4°C for 16 hours. After, the solution was centrifuged (6000g, 10 min, + 4°C), the supernatant was removed, and the precipitate was washed three times with cooled 90% alcohol. The precipitate was dried at a temperature of +50°C.

### Determination of the dependence of xylanase activity on temperature and pH

The xylanase activity was determined by the amount of reducing sugars (in terms of xylose) formed from a 1% xylan solution per unit of time at pH 6.5 and a temperature of +50°C [6].

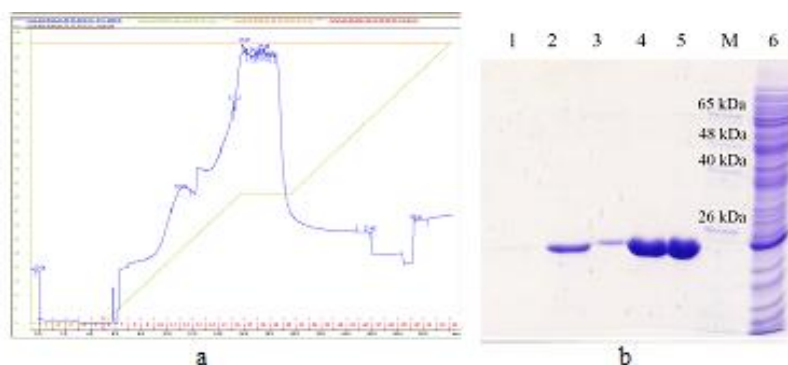
Determination of the dependence of activity on the temperature in the range from +25 to 80°C, and on pH in the range of 2.7-10.2.

## RESULTS AND DISCUSSION

The xylanase gene *xynA* was cloned as part of the genetic engineering construct pET-28c(+)/XynA. In the open reading frame, there is a protein with 197 amino acid residues with deleted signal peptide and a calculated mass of 21.8 kDa. The recombinant protein contains a hexahistidine tag at the C-end.

Comparison of the obtained gene sequence with the reference sequence of the xylanase carbohydrase gene from the NCBI database (AF441773.1) showed the presence of 9 nucleotide substitutions, 1 from which leads to amino acids substitution in the target protein: Lys<sup>295</sup>→Tyr<sup>295</sup>.

Figure 1 shows the chromatogram and electrophoregram of XynA protein purification:



1 –flush the column buffer 20 mM Tris-HCl (pH 8,0), 500 mM NaCl, 350 mM Imidazole, 2,4,5 - chromatographic fractions № 18-20, 3 - chromatographic fraction №21, 6 - load  
Fig. 1. The chromatogram of protein purification XynA (a) and protein electrophoresis fractions (b)

As follows from the figure 1, tagged protein XynA eluted from the column at an imidazole concentration of 232 mM. Electrophoresis of the fractions corresponding to the chromatographic

peak showed that the fractions 19-21 contain xylanase with electrophoretic purity. The concentration of xylanase in fraction 20 is 2 mg/ml.

The xylan obtained from beech sawdust has a powdery form of a light cream shade. In cold water, xylan swells like starch and similar polymers. The yield of xylan was 11 g from 200 gram of sawdust.

During this study, the dependence of the enzymatic activity of recombinant xylanase from temperature and pH has shown the highest activity at +50°C and a pH of 6.9. The activity at this temperature and pH was 1859 units per ml of purified protein. The working temperature and pH ranges in which xylanase conserves more than 70% activity is 40-60°C and 5-8, respectively.

## CONCLUSION

Thermal stability of xylanase is an important parameter since high temperatures are used in papermaking, baking and feed manufacturing. Xylanase from the soil bacterium *Bacillus licheniformis* shows a sufficient level of thermal stability (up to +50°C) and tolerance to changes in pH (5-8), which makes this enzyme perspective for industrial application. The maximum activity of recombinant carboxydase xylanase from *B.licheniformis* is 930 units/mg.

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## ***BACILLUS LICHENIFORMIS* ҚҰРАМЫНАН РЕКОМБИНАНТТЫ КАРБОГИДРАЗА КСИЛАЗАНЫ БӨЛІП АЛУ ЖӘНЕ ЗЕРТТЕУ**

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### **ТҮЙІН**

Ксиланазалар өсімдік биомассасының негізгі компоненті болып табылатын ксиланның деградациясына қатысатын гидролитикалық ферменттерді білдіреді. Ұсынылып отырған жұмыста қазақстандық *Bacillus licheniformis* штаммынан алынған хупА ксиланазасы *E.coli* жасушаларында рекомбинантты ақуыз түрінде алынды. ХупА гені *B.licheniformis* геномдық ДНҚ-нан амплификацияланып, T7 бактериофагы промоторының бақылауындағы рЕТ-28с (+) векторына клондалды. Рекомбинантты ХупА ақуызы ArcticExpressRP(DE3) жасушаларында плазмидті экспрессия арқылы алынды, ақуыз аффинді хроматография тәсілімен тазаланды. Рекомбинантты ксиланазаның ферментативті белсенділігінің температураға және рН көрсеткіштеріне тәуелділігін зерттеу барысында келесі мәліметтер алынды, ксиланаза +50°C және рН 6,9 көрсеткіштерінде максималды белсендікке ие болады. Температура мен рН осындай көрсеткіштерінде белсендік сәйкесінше 1859 бірліктерді құрады. Ксиланазаның белсенділігінің барынша жоғары көрсеткішін 70% аса дәрежесінде сақтай алатын жұмыс температурасы мен рН көрсеткіштері 40-60°C және 5-8 тең. Алынған деректердің өсімдік материалдарын қайта өңдеу кезінде биотехнологиялық үрдістерінде рекомбинантты ксиланаза карбогидразаны пайдалану үшін маңызы зор.

Негізгі сөздер: ксиланаза, *Bacillus*, рекомбинантты фермент, ксиланазалық белсенділік.

## **ПОЛУЧЕНИЕ И ИЗУЧЕНИЕ РЕКОМБИНАНТНОЙ КАРБОГИДРАЗЫ КСИЛАЗАЗЫ ИЗ *BACILLUS LICHENIFORMIS***

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## АБСТРАКТ

Ксиланазы представляют собой гидролитические ферменты, участвующие в деградации ксилана, основного компонента растительной биомассы. В представленной работе ксиланаза XynA из казахстанского штамма *Bacillus licheniformis* была получена в рекомбинантной форме в клетках *E.coli*. Ген xynA был амплифицирован из геномной ДНК бактерии *B.licheniformis* и клонирован в составе вектора pET-28c (+) под контролем промотора бактериофага T7. Путем плазмидной экспрессии гена в клетках *E.coli* ArcticExpress RP(DE3) получали рекомбинантный белок XynA, очистка которого проводилась методом металло-аффинной хроматографии. При изучении зависимости ферментативной активности рекомбинантной ксиланазы от температуры и pH было установлено, что ксиланаза имеет максимум активности при + 50°C и pH 6,9. Активность при этих значениях температуры и pH составила 1859 единиц на мл фракции очищенного белка. Диапазон рабочих температур и pH, в которых ксиланаза сохраняет активность более 70% от максимальной, составляет 40-60°C и 5-8, соответственно. Полученные данные являются существенными для использования рекомбинантной карбогидразы ксиланазы в биотехнологических процессах при переработке растительного сырья.

Ключевые слова: ксиланаза, *Bacillus*, рекомбинантный фермент, ксиланазная активность